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Microbial community activities during establishment, performance, and decline of bench-scale passive treatment systems for mine drainage

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Abstract

Permeable reactive barrier (PRB) technology, in which sulfate-reducing bacteria (SRB) facilitate precipitation of metal sulfides, is a promising approach for remediation of sulfate- and metal-laden mine drainage. While PRBs are easily established, they often decline for reasons not well understood. SRB depend on or compete with multiple dynamic microbial populations within a PRB; as a result, performance depends on the changing PRB chemical composition and on succession and competition within the microbial community. To investigate these interactions, we constructed and monitored eight bench-scale PRBs to define periods of establishment, performance, and decline. We then conducted short-term batch studies, using substrate-supplemented column materials, on Days 0 (pre-establishment), 27 (establishment), 41 (performance), and 99 (decline) to reveal potential activities of cellulolytic bacteria, fermenters + anaerobic respirers, SRB, and methanogens. PRBs showed active sulfate reduction, with sulfate removal rates (SRR) of ~1–3 $mol/m^3/d$, as well as effective removal of Zn^{2+}. Potential activities of fermentative + anaerobic respiratory bacteria were initially high but diminished greatly during establishment and dropped further during performance and decline. In contrast, potential SRB activity rose during establishment, peaked during performance, and diminished as performance declined. Potential methanogen activity was low; in addition, SRB-methanogen substrate competition was shown not to limit SRB activity. Cellulolytic bacteria showed no substrate limitation at any time. However, fermenters experienced substrate limitation by Day 0, SRB by Day 27, and methanogens by Day 41, showing the dependence of each group on upstream populations to provide substrates. All potential activities, except methanogenesis, were ultimately limited by cellulose hydrolysis; in addition, all potential activities except methanogenesis declined substantially by Day 99, showing that long-term substrate deprivation strongly diminished the intrinsic capacity of the PRB community to perform.

Keywords: Permeable reactive barrier; Sulfate reduction; Microbial community; Mine drainage; Groundwater treatment

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1. Introduction

Mine drainage is caused by oxidation of iron and sulfide minerals in ores exposed to air and water, yielding an aqueous stream with high levels of dissolved toxic heavy metals and sulfate (Evangelou, 1995). Thousands of remote, abandoned mines generate metal-laden drainage throughout the western United States, yet traditional remediation techniques are too expensive to be employed at these sites (US Environmental Protection Agency, 1995). Cost-effective, low-maintenance, long-term approaches for removal of metals and sulfate are therefore greatly desired, and passive treatment wetlands and barriers have emerged as technologies with great promise (Eccles, 1999; Blowes et al., 2000; Herbert et al., 2000; Kalin, 2001). These anaerobic passive treatment systems support the activity of sulfate-reducing bacteria (SRB), which generate metabolic energy by reducing sulfate to sulfide using simple organic substrates or H_2 as electron donors. Dissolved sulfide then precipitates soluble metals as metal sulfides, which typically possess low solubilities over a wide pH range and are therefore retained in the barrier (Hammack and Edenborn, 1992; Fortin et al., 1994; Eccles, 1999).

Permeable reactive barriers (PRBs) are passive treatment systems designed to intercept mine drainage belowground and typically consist of channels filled with inexpensive organic substrates (wood chips, crop waste, compost, manure, sawdust, etc.) blended with gravel to enhance permeability and limestone or bicarbonate to provide alkalinity (Waybrant et al., 1998; Herbert et al., 2000; Benner et al., 2002). PRB performance is often excellent in the first months after establishment (Wildeman et al., 1997; Wildeman and Updegraff, 1997; Cocos et al., 2002), but longer-term performance in both field and laboratory-scale systems consistently declines well before the theoretical capacities of the barriers have been reached (Webb et al., 1998; Blowes et al., 2000). Primary explanations offered for this phenomenon include depletion of the reactive component of the barrier, decline of reactive surfaces resulting from mineral precipitation, clogging or channeling within the barrier, low seasonal temperatures, and low influent pH values (Blowes et al., 2000; Benner et al., 2002; Waybrant et al., 2002). Although each of these is important under certain conditions, the first, ''depletion of the reactive component'', appears to be the most universally important and also the least-understood (Wildeman et al., 1997; Wildeman and Updegraff, 1997; Waybrant et al., 2002).

As the ''reactive component'', a variety of cellulosic organic substrates have been used to support microbial activity in anaerobic passive treatment systems (Waybrant et al., 1998; Gilbert et al., 1999; Chang et al., 2000; Cocos, 2002, Gibert et al., 2004), under the assumption that cellulose would provide a long-term source of carbon and energy to sustain the sulfate-reducing population (Cocos, 2002). Additionally, the SRB were expected to require sufficient sulfate, nutrients (N, P, vitamins, etc.), and near-neutral pH values (Waybrant et al., 1998).

Only a small fraction of the organic matter placed in passive treatment systems is likely to be immediately available to SRB (Benner et al., 1999), because these microorganisms typically require simpler molecules (organic acids, alcohols, or H_2) for energy (Widdel, 1988). Anaerobic degradation of complex material to simpler compounds by cellulolytic and fermentative microbes is therefore required (Colberg, 1988; Widdel, 1988; Wildeman and Updegraff, 1997) and may limit the rate at which substrates become available to SRB. The activities of the cellulolytic and fermentative bacteria are consequently vital to the long-term sustainability of passive treatment systems, as they are to the energy flow in many natural anaerobic systems (Lynd et al., 2002). Their importance to the sulfate-reduction rate itself remains unclear, however, because sulfate reduction is likely to represent such a small component of the total carbon and energy flow in the system (Capone and Kiene, 1988) and is vulnerable to so many other factors. In particular, concerns have been raised that SRB activity may suffer from nitrogen, phosphorus, and/or sulfate limitation (Waybrant et al., 2002), as well as from competition from methanogens for H_2, acetate, formate, or other competitive substrates, diverting energy away from sulfide production (Daly et al., 2000).

To elucidate the interactions of microbial communities during PRB operation, therefore, we investigated the potential activities over time of four important microbial groups: cellulolytic microbes, anaerobic respirers + fermenters, sulfate-reducers, and methanogens, throughout the lifetimes of eight bench-scale passive treatment systems. The microbial activities we sought to probe, among the many possible, are shown in Fig. 1. Functional bench-scale passive treatment columns were constructed from typical materials (alfalfa, manure, wood chips, limestone, sand) and monitored for active sulfate and metal removal from simulated mine drainage. At intervals of several weeks, pairs of columns were sacrificed, divided into batches, and supplemented individually with cellulose and four of its successive degradation products: cellobiose, glucose, lactate, and acetate. By monitoring microbial community responses to the supplements in terms of CO_2 production (indicating overall organic carbon oxidation), H_2S production (indicating sulfate reduction), and CH_4 production (indicating methanogenesis), the potential of each microbial community for activity, as well as the limiting substrates for each process, were revealed throughout the lifetimes of the columns. Concentrations of H_2, representing an important energy flow pathway in

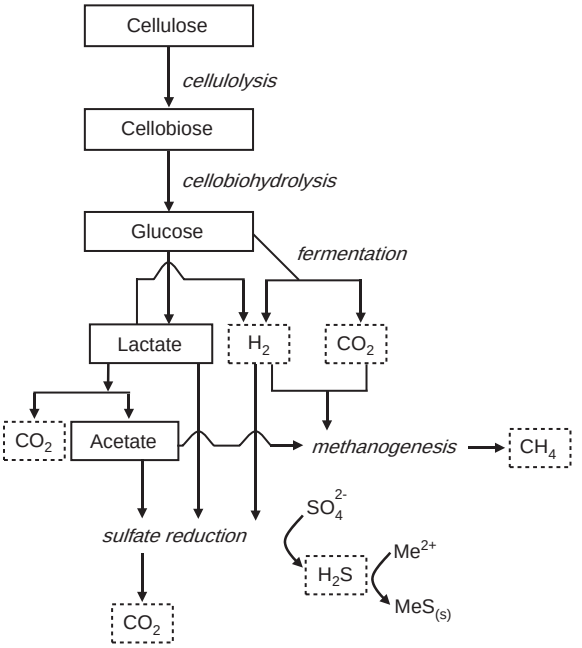

Fig. 1. Diagram of microbial processes under investigation. Solid boxes designate organic substrates added to batch studies; dashed boxes indicate measured metabolic products.

anaerobic microbial communities (Lovley and Goodwin, 1988; Lovley et al., 1994) as well as a point of competition between methanogens and sulfate reducers (Qatibi et al., 1990; Raskin et al., 1996; O'Flaherty et al., 1998), were also monitored.

The ability of a supplement and its degradation products to stimulate a particular activity, combined with the inability of more complex supplements to stimulate the same activity, was designated as evidence that the conversion step between the most complex stimulatory substrate and the least complex non-stimulatory substrate limited the activity of interest.

2. Materials and methods

2.1. Column specifications

Eight glass columns, of 5 cm ID × 30 cm height, each with four vertical sampling ports, were employed. Sampling ports were sealed with rubber gaskets and screw caps, and column bottoms were sealed with Delrin™ plastic caps and rubber o-rings. A stainless steel screen was placed in the bottom of each plastic cap to prevent loss of organic material. Rubber stoppers (#11), each with a 1/8″ glass tube inserted, were used to seal the tops of the columns. Each glass tube was connected via 1/8″ Tygon tubing to a one-way check valve, which allowed gas to escape the columns while preventing air from entering. Influent was pumped via a 12-channel peristaltic pump (Ismatec IPC) in an upflow configuration, entering each column through Luer lock fittings on the Delrin end caps and exiting through Luer lock fittings, secured by o-rings and screw caps, within the sampling ports located 15 cm above the bottom. The static water level was maintained above the effluent ports to facilitate maintenance of anaerobic conditions.

2.2. Column packing

Columns were packed with 150 g dry weight of a homogenized mixture typical of laboratory and field-scale passive treatment systems in its abundant organic carbon and nitrogen, inert material for porosity enhancement, and source of alkalinity (Waybrant et al., 1998, 2002): 15% walnut wood shavings, 20% dairy cattle manure, 10% alfalfa pellets, 5% wetland sediment (Big Five treatment wetland, Idaho Springs, CO), 5% crushed limestone rock (#10–#20 mesh; Pioneer Sand Co., Golden, CO), and 45% #8 mesh silica sand. Walnut shavings and alfalfa pellets were processed to 4 mm in a Wiley mill. Columns were packed to a depth of 15.5 cm under deionized water and allowed to settle for 1 day before influent began, yielding a final reactive volume of 304 cm^3. The remaining space in each column was filled with silica sand, separated from the organic layer by a fine mesh metal screen to minimize loss of organic material.

2.3. Influent composition

Column influent consisted of 1000 mg/L sulfate as Na_2SO_4, pH 6.0, at 90 mL/d initially to allow sulfate reduction to be established. Influent alkalinity was approx. 1.3 mg $CaCO_3$/L. On Day 24, for six of the eight columns, the sulfate influent was supplemented with 50 mg/L of manganese as $MnSO_4 \cdot H_2O$ and zinc as $ZnSO_4 \cdot 7H_2O$ to simulate the mine drainage in St. Kevin Gulch, Leadville, CO (Smith, 1991), and influent flow rate was diminished to 30 mL/d. The remaining two columns were kept under initial conditions until their sacrifice on Day 27. On Day 29, the influent sulfate concentration was increased to 2000 mg/L for a period of 8 days, after which it was decreased to 1000 mg/L.

2.4. Effluent collection and analysis

Effluent was collected in plastic bottles open to the atmosphere and monitored for sulfate, total sulfur and metals, pH, alkalinity, and conductivity. Sulfate reduction rates (SRRs) were calculated as: SRR (mol SO_4^{2-}/m^3/d) = influent flow (L/d) [influent SO_4^{2-} (M)−effluent SO_4^{2-}(M)] [packed volume (m^3)]$^{-1}$.

2.5. Batch experiments

Gases are sensitive indicators of microbial activity but are difficult to sample accurately in flow-through systems. Therefore, to assess individual microbial activities, we employed multiple replicate columns, sacrificed them at timepoints of interest as indicated by effluent analysis, and conducted batch investigations with column materials over time scales sufficiently short to prevent significant community change.

On Days 0, 27, 41, and 99, pairs of columns were disconnected and contents were homogenized and subdivided into 12 160 mL serum bottles (six replicate pairs) for substrate amendment experiments. Each bottle contained 10 g dry weight of column material and 85 mL liquid, consisting of 1000 mg/L sulfate as Na_2SO_4 and 10 mM of one of the following: cellulose (as glucose monomers) (microcrystalline; Supelco #5-8027), cellobiose (Sigma), glucose (Aldrich), sodium lactate (Sigma), or sodium acetate (Sigma). Substrate supplements were omitted from negative controls. Slurries were sparged with high-purity N_2 for 20 min and headspaces were sparged for 7 min, after which bottles were sealed with thick rubber stoppers and aluminum crimp tops. The pH of the mixtures in the bottles was not modified by external acid/base addition initially or at any time during the experiment.

Serum bottles were incubated on their sides at room temperature, shaking at 60 rpm, for 60 h. Liquid and gas samples were taken using sterile, anoxic technique at 0, 6, 12, 24, 30, 36, 48, 54, and 60 h, removing 1.5 mL of gas and 3 mL of liquid at each sampling. Syringes carrying gas samples were fitted with Luer-lock Mininert syringe valves and analyzed immediately for CO_2, CH_4, H_2S, and H_2.

The batch experiment conditions were chosen to facilitate direct comparison of responses of the existing microbial communities to substrate amendments. Specifically, the relatively short incubation period was selected to reveal consistent metabolic activity patterns without a significant change in the microbial population, such that the no-growth form of the Monod model could be used to approximate the rate of substrate consumption (Alexander, 1999):

$$-\frac{dS}{dt} = \frac{kS}{K_s + S}, \quad (1)$$

where S is the (organic) substrate concentration, k is a composite rate constant that incorporates the effects of microbial population density, and K_S is the Monod constant. For the relatively high substrate concentrations provided by each amendment (10 mM), S can be assumed to be much larger than K_S and thus:

$$\frac{-dS}{dt} \cong k. \quad (2)$$

Literature reports of mixed anaerobic culture kinetics include K_S values of 0.1–2.2 mM for glucose (Pavlostathis and Giraldo-Gomez, 1991; Gavala and Lyberatos, 2001), 0.1–5.2 mM for lactate (Pavlostathis and Giraldo-Gomez, 1991; Omil et al., 1998), and 0.8–0.9 mM for acetate (Omil et al., 1998; Gavala and Lyberatos, 2001). Since the values of K_S are specific to a particular culture, the validity of the two assumptions used here is best assessed by examining the data from the batch experiments. The rate of formation of the direct metabolic products (CO_2, H_2S, etc.) is expected to be directly proportional to $(-dS/dt)$ and zero-order with respect to S, and thus plots of product concentration vs. time should be linear. The trends in Figs. 4–7 are in agreement with this prediction and thus these results can be used to compare rates of substrate uptake directly.

2.6. Analytical methods

Sulfate was quantified by ion chromatography (Dionex ICS-90, AS14A column, CO_3/HCO_3 eluent with 1 mL/min flow rate). Samples were filtered through 0.2-μm syringe-tip filters, stored for up to 5 d at 4 °C, and diluted with Milli-Q water before analysis. The detection limit for sulfate was 7.5 mg/L (0.078 μM). Total sulfur and metals were analyzed by inductively coupled plasma absorbance emission spectroscopy (ICP-AES) (Perkin Elmer Optima 3000). Samples were filtered through 0.2-μm syringe-tip filters, diluted with Milli-Q water, and acidified to pH 2 with trace metals-grade nitric acid (Mallinckrodt). Detection limits were 0.05 mg/L, 0.7 μg/L, and 2.3 μg/L for sulfur, Mn, and Zn, respectively. Alkalinity was measured by Hach digital titration, method #8203, and conductivity was measured with a YSI 35 probe and meter. CO_2, H_2S, CH_4, and H_2 were quantified by gas chromatography (Agilent P200 Micro GC with thermal conductivity detector, He carrier gas, Pora Plot U 8-m column, 100 °C). Detection limits were 0.15% for CO_2, 0.01% for H_2S, 0.03% for CH_4, and 0.01% for H_2. Instruments were calibrated with four or more standards encompassing the range of experimental data before each use. During IC and ICP analyses, one set of standards, one blank, and one duplicate sample were analyzed after every 10 experimental samples; during GC analyses, one blank and one set of standards were analyzed after every 12 experimental samples.

2.7. Calculation of sulfate removal rates

SRRs were obtained by comparing the measured effluent sulfate concentrations with those expected in the absence of sulfate reduction or sorption. These SRRs were expressed per unit volume of empty column (304 cm^3). Since the influent sulfate concentration changed during the column operation, the program

CXTFIT (Toride et al., 1999) was used to calculate effluent sulfate concentrations assuming an absence of microbial reduction and assuming conservative transport. CXTFIT was first used to estimate the dispersion coefficient D (6.28 cm^2/d) and the average linear velocity v (2.26 cm/d when the influent rate was 30 cm^3/d) based on the conductivity changes that accompanied the 8-day rise in influent sulfate concentration that began on Day 29. Since the exact time at which sorption ceased to be a removal mechanism could not be pinpointed, sulfate reduction rates were approximated by sulfate removal rates (Waybrant et al., 2002). SRR were determined by subtracting measured effluent sulfate concentrations from influent sulfate concentrations modeled considering only conservative transport, multiplying this quantity by the volumetric flow rate, and dividing by the empty column volume (304 cm^3). The estimated mean residence time of the columns was 6.9 d when the influent rate was 30 cm^3/d, corresponding to a pore volume of 206 cm^3.

2.8. Statistical methods

Gas production data were analyzed using a mixed linear model with repeated measures. The batch study supplement was the main effect, with time (continuous) as a covariate, and with duplicates nested within the main treatment effect. Treatment differences within batch studies during each time period were analyzed using one-way ANOVAs, with batch supplement as the treatment and gas production as the response. Treatment differences between batch studies 1, 2, 3, and 4, in terms of response to each supplement, were found using Student's t-tests, and differences in gas production among supplements within each batch study were analyzed using the Bonferroni test of means (Scheiner, 1993). A significance level of $\alpha = 0.05$ was used for all statistical tests. All analyses were performed using Minitab Release 14 Statistical Software (Minitab Inc., State College, PA).

3. Results and discussion

3.1. Column performance

3.1.1. Sulfate removal

Sulfate was introduced to the columns at 1000 mg/L beginning on Day 0. During the establishment period, effluent sulfate increased as the sulfate traveled through the columns (Fig. 2a) and reached approx. 900 mg/L in a local maximum on Day 10; this maximum suggested that the sorption capacity of the columns for sulfate had been reached. Subsequent decline of effluent sulfate and increase of SRR (Fig. 2b) indicated that sulfate reduction had become established within 3 weeks of column packing. This observation was consistent with previous studies involving either batch experiments or continuous-flow reactors (Lyew et al., 1994; Waybrant et al., 1998; Cocos, 2002). After 27 d of operation, the six remaining columns showed a mean SRR of 1.8 ± 0.2 mol/m^3/d, beginning the "performance" period. From Days 29–37, influent sulfate was inadvertently increased from 1000 to 2000 mg/L. A corresponding increase in effluent sulfate was observed (Fig. 2a), accompanied by a drop in average SRR to 0.6 ± 0.3 mol/m^3/d (Day 34) before recovery began. This indicated that sulfate reduction in the columns was not limited by influent sulfate level, consistent with the sulfate half-saturation constant values of 20–160 mg/L typically reported for SRB (Roychoudhury et al., 2003; Roychoudhury, 2004).

Influent sulfate was returned to 1000 mg/L on Day 37, and effluent concentrations in all columns diminished by Day 71 to levels near or below their Day 27 values. Not surprisingly, given the complexity of the systems, columns showed varying responses to the sulfate pulse: the SRR of column 5 was greater on Day 52 than on Day 27, removing sulfate to levels below 100 mg/L, and the SRR of column 6 was also greater by Day 66 than it had been before the sulfate pulse. Columns 3 and 4, in contrast, reached but did not exceed SRRs comparable to their Day 27 values (Fig. 2b).

The average SRR among the columns was 1.8 ± 0.2 mol SO_4^{2-}/m^3/day on Day 27, when the first pair (2 and 8) was sacrificed, and recovered to this level (1.8 ± 0.6 mol SO_4^{2-}/m^3/day) by Day 66 in the performance period. By Day 99, however, when the third pair (3 and 4) was sacrificed, the average SRR of the four remaining columns had diminished significantly ($p < 0.001$) to 1.3 ± 0.60 mol SO_4^{2-}/m^3/day, showing that the period of decline in sulfate-reducing activity had begun and indicating that the corresponding batch studies were therefore of special interest. All sulfate reduction rates were consistent with rates reported by Wildeman and colleagues in a pilot reactor treating mine drainage (0.3–2.0 mol S^{2-} produced/m^3/day) (Wildeman et al., 1997) and with those of Waybrant et al. in laboratory-scale columns (0.7 mol SO_4^{2-} removed/m^3/day) (Waybrant et al., 2002).

3.1.2. Alkalinity production

Substantial alkalinity was generated by the columns throughout their lifetimes, presumably as a consequence of both bicarbonate dissolution and of microbial activity. Influent alkalinity was negligible at approx. 1.3 mg $CaCO_3$/L. Effluent alkalinity reached quite high levels (~2000 mg $CaCO_3$/L) initially but rapidly declined to values between 600 and 1000 mg $CaCO_3$/L for each column; these values were then maintained with some variation but without substantial decline throughout the lifetime of the columns (data not shown) (Logan, 2003).

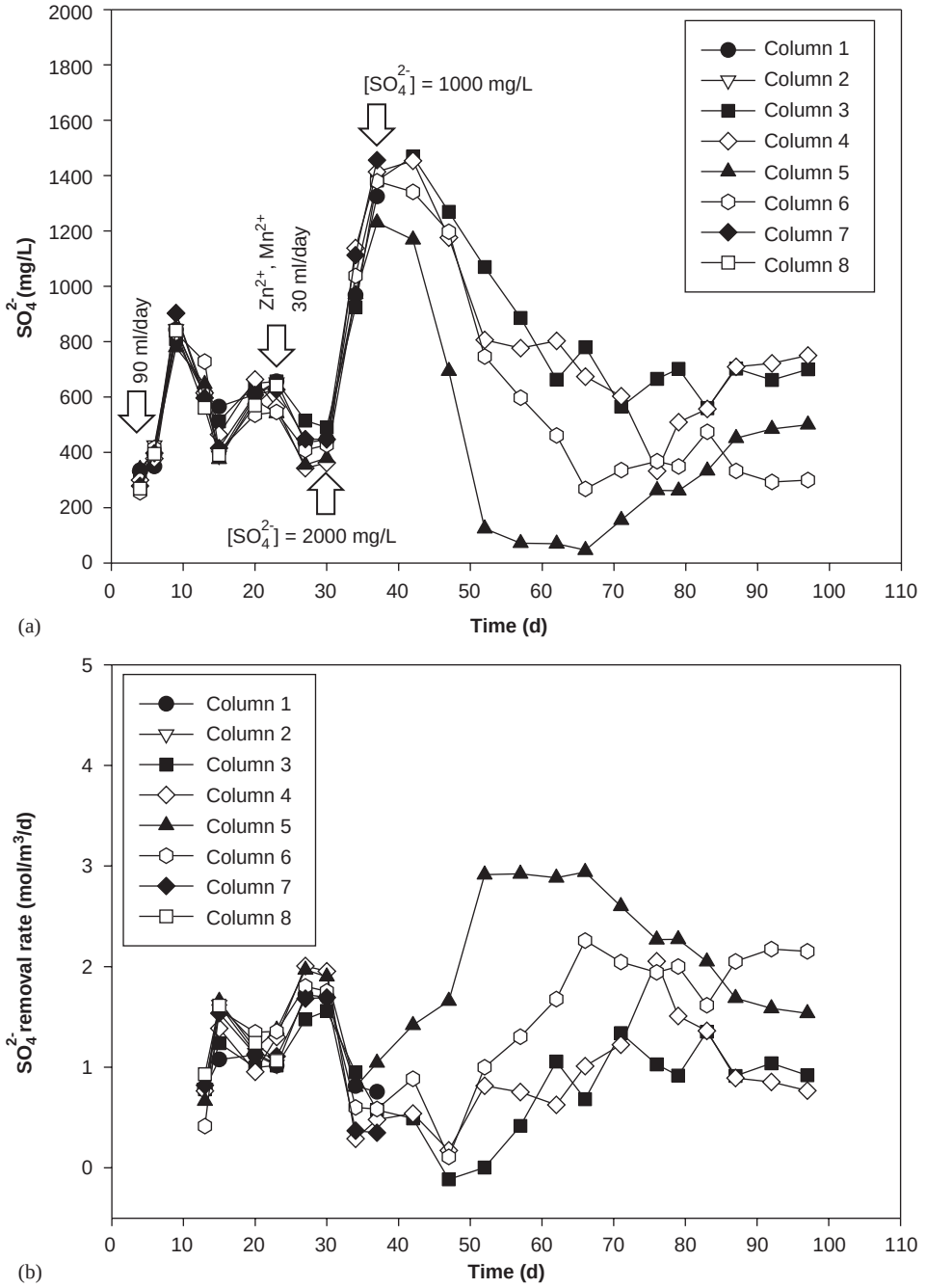

Fig. 2. Effluent sulfate concentrations (a) and sulfate removal rates (b) over time from bench-scale passive treatment columns. Influent sulfate levels were 1000 mg/L from Days 0 to 29, 2000 mg/L from Days 30 to 37, and 1000 mg/L from Days 38 to 99. On Day 24, Zn and Mn were added to the influent at 50 mg/L each. Open arrows (a) show timepoints of changes and apply to (b) as well. Data points show individual measurements.

Consistent with the alkalinity results, column effluent pH gradually rose throughout the 14-week lifetimes of the columns from approx. 6.5 to approx. 7.5, showing that the influent composition (pH = 6.0) was modified appreciably (data not shown) (Logan, 2003). This pH change would have enhanced the precipitation of carbonate and hydroxide minerals, as well as sulfides (discussed below), but was not expected to compromise

the activity of SRB, which have a reported pH tolerance of <5 to 9.5 (Postgate, 1979).

3.1.3. Metal removal

Effluent metals were monitored to assess column performance. From Days 0 to 24, column influent was metal-free, and the small amounts of Zn and Mn observed in the effluent therefore originated in column packing materials. Beginning on Day 24, indicated by open arrows in Figs. 2a and b, the influent was supplemented with 50 mg/L each of Mn and Zn. Zinc was removed to levels below the detection limit (BDL; $<2.3\,\mu g/L$) by all columns throughout their lifetimes (Fig. 3), presumably by precipitation of $ZnS_{(s)}$: this solid was greatly supersaturated in every column at all times, based on the assumption that at least 10% of the sulfate removed from the influent had been converted to sulfide. This result was consistent with results of Lyew and Sheppard showing that Zn is among the metals most easily removed in sulfate-reducing systems (Lyew and Sheppard, 1997). While $ZnCO_{3(s)}$ was also potentially saturated in every column throughout its lifetime (assuming influent Zn levels, bicarbonate as the predominant source of alkalinity, and $K_S = 10^{-10.0}$ (Morel and Hering, 1993)), at no time could $ZnCO_{3(s)}$ precipitation have controlled Zn levels to the BDL concentrations observed. $Zn(OH)_{2(s)}$ was also saturated for some columns at some times, but also could not have controlled Zn levels to those observed.

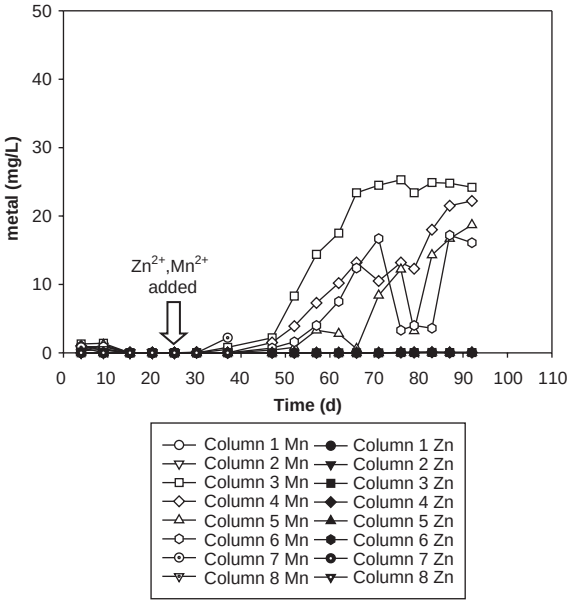

Fig. 3. Effluent Zn and Mn concentrations over time from bench-scale passive treatment columns. Influent concentrations were 0 mg/L for Days 0–23 and 50 mg/L for Days 24–99 (open arrow). Data points show individual measurements.

As a result, $ZnS_{(s)}$ precipitation is by far the most plausible explanation for the observations.

Greater than 90% of influent Mn was removed during the first several weeks, potentially due, at least in part, to sorption onto the column materials, which had sorption capacities equivalent to several hundred mg Mn per packed column (Seyler, 2003). $MnS_{(s)}$ was saturated in all columns throughout their lifetimes (assuming that at least 10% of the sulfate removed from the influent had been converted to sulfide, and $K_S = 10^{-10.5}$ (Morel and Hering, 1993)), as was $MnCO_{3(s)}$ (assuming influent Mn levels, bicarbonate as the predominant source of alkalinity, and $K_S = 10^{-9.3}$ (Morel and Hering, 1993)), and their precipitation could also have contributed to Mn removal. After Day 46, however, Mn appeared in increasing concentrations in the effluents of all columns (Fig. 3). This was not unexpected, based on previous reports that have demonstrated requirements of high pH values (8–10) for rapid formation of MnS and $MnCO_3$ precipitates (Wildeman and Updegraff, 1997; Clayton et al., 1999). Columns showed diverse Mn removal abilities during this time, with greatest Mn removal found in columns with greatest sulfate reduction (e.g. Column 5) and least Mn removal found in columns with least sulfate reduction (e.g. Column 3) (Pearson correlation of sulfate and Mn removal of Columns 3–6 over time = 0.435, $p = 0.002$). This observation suggests that $MnS_{(s)}$ precipitation continued to contribute to Mn removal, and/or that the greater alkalinity found in columns with greater sulfate reduction may have facilitated continued $MnCO_{3(s)}$ precipitation.

3.1.4. Batch supplement studies

Pairs of columns were sacrificed at intervals and column contents were supplemented individually with cellulose, cellobiose, glucose, lactate, and acetate in batch experiments. Production of CO_2, H_2S, CH_4, and H_2 was monitored to assess microbial responses. The initial pH of the slurries in each of the batch experiments was 6.5, except for those bottles that had been amended with acetate or lactate, in which cases the initial pH was 4.5. After 60 h, the pH in all bottles was in the range 6.3–7.1. Thus, pH differences were consistent across the different batch studies. The lower initial pH in the acetate- and lactate-amended bottles does not appear to have been a significant factor since the initial production of CO_2 buffered the acidity early in the experiment.

3.1.4.1. H_2 production. H_2 concentrations were below the detection limit in all samples (data not shown), presumably due to rapid consumption by anaerobic respirers, including SRB (Qatibi et al., 1990; Raskin et al., 1996; O'Flaherty et al., 1998; Schwartz and Friedrich, 2003). The role of H_2 in energy dynamics within passive treatment systems remains a topic of great importance, however, and limitation of H_2

production may have contributed to many of the substrate-limitation results presented below.

3.1.4.2. Initial study. In the initial (Day 0) batch study, using fresh column materials that had not yet been exposed to sulfate or metals, headspace CO_2 concentrations ranged from 31.8% (no supplement) to 62.5% (cellobiose) after 60 h of incubation (Fig. 4a). Considerable CO_2 was produced by unsupplemented column materials, particularly in comparison to subsequent batch studies, indicating that the column mixture itself possessed readily oxidizable organic carbon.

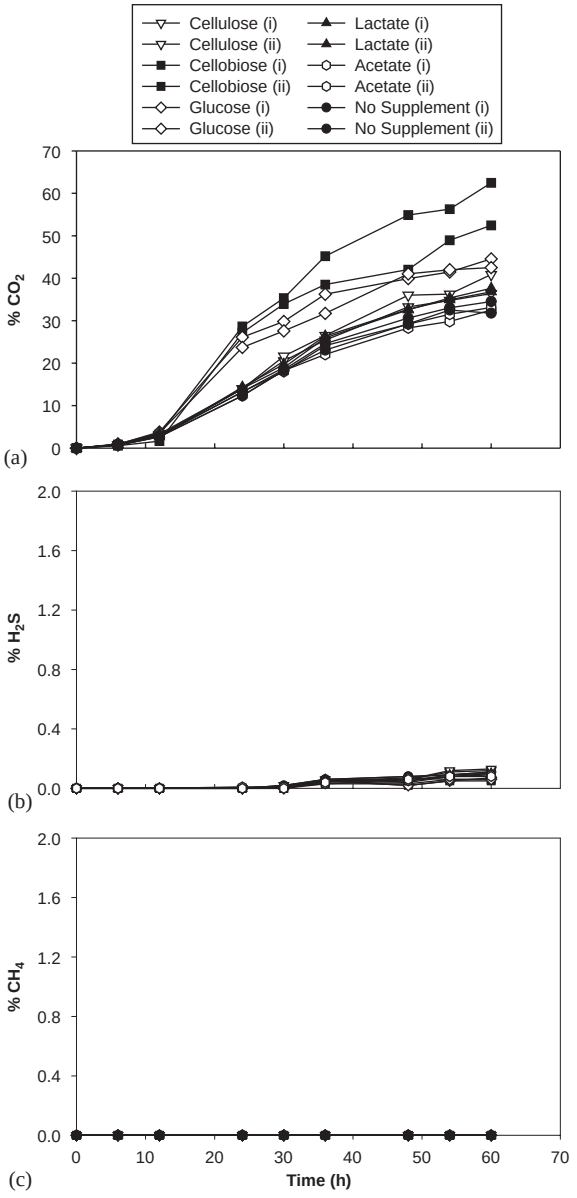

Fig. 4. Headspace molar percentages of (a) CO_2, (b) H_2S, and (c) CH_4 produced over time in Day 0 batch studies. Replicate batches consisted of column packing materials supplemented with 10 mM cellulose (as glucose subunits), cellobiose, glucose, lactate, or acetate; controls received no supplements. Data points show individual measurements.

Among the supplements tested, cellobiose, glucose, and lactate stimulated greatest and nearly equivalent CO_2 production (cellobiose = glucose, $p = 0.159$; glucose = lactate, $p = 0.602$; cellobiose > acetate, $p = 0.033$). Acetate supplementation led to production of $32.8 \pm 0.3\%$ headspace CO_2, indistinguishable from unsupplemented controls ($p = 0.976$); cellulose supplementation also produced CO_2 indistinguishable from controls ($p = 0.668$).

The abilities of cellobiose and glucose to stimulate heterotrophic metabolism indicated that availability of these substrates already limited the most active members of the microbial community, potentially including fermenters, facultative anaerobes, and other microbes able to metabolize sugars and disaccharides.

Among the substrates that did not stimulate CO_2 production, cellulose was likely to have been less available to the microbial communities due to its stable, semi-crystalline structure (Lynd et al., 2002). Lactate and acetate, in turn, are readily bioavailable compounds, but are used most actively under anoxic conditions by respirers such as sulfate-reducers and methanogens (Schlegel, 1993). These groups were clearly minimally active, as expected given the very brief incubation of the column packing materials under anoxic conditions.

Sulfate reduction yielded $\leqslant 0.1\%$ headspace H_2S in the Day 0 study after 60 h, with supplemented batches showing sulfide production indistinguishable from that of unsupplemented controls ($p = 0.139$) (Fig. 4b). Methane production was below the detection limit in all samples, showing that supplementation failed to stimulate this activity as well (Fig. 4c). The extremely low productions of sulfide and methane were expected in this initial study, however, again because of the limited exposure of column materials to anoxic conditions.

3.1.4.3. Second study. Columns 2 and 8, sacrificed for the second (Day 27) batch study, were actively reducing sulfate ($SRR = 1.8 \pm 0.2\ mol\ SO_4^{2-}/m^3/day$; Fig. 2b) but had not yet been exposed to metals. In this study, CO_2 production was significantly ($p < 0.001$) lower in all samples than observed in the initial study (cf. Figs. 4a and 5a). Relevant rate comparisons are also shown graphically in Figs. 8a–c (CO_2, H_2S, and CH_4, respectively) described below. Unsupplemented controls reached headspace CO_2 concentrations of only $2.07 \pm 0.01\%$ (Fig. 5a), indicating that easily oxidizable organic carbon within the column mixtures had been substantially (~90%) depleted within the first 4 weeks. This phenomenon reflected previous observations that labile organic carbon is rapidly consumed in passive

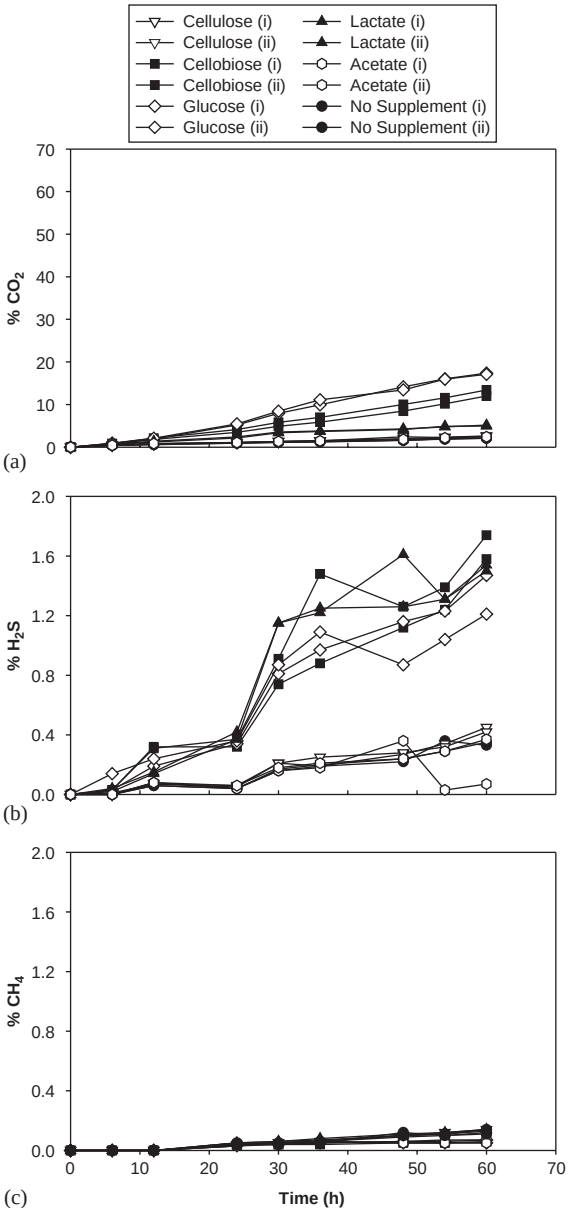

Fig. 5. Headspace molar percentages of (a) CO_2, (b) H_2S, and (c) CH_4 produced over time in Day 27 batch studies. Replicate batches consisted of sacrificed column materials supplemented with 10 mM cellulose, cellobiose, glucose, lactate, or acetate; controls received no supplements. Data points show individual measurements.

mine drainage treatment systems (Tsukamoto and Miller, 1999). Labile carbon depletion has also frequently accompanied declines in performance (Tsukamoto and Miller, 1999), highlighting the need to understand which substrate(s) and/or competing communities directly limit SRB activity.

CO_2 production was stimulated most by glucose ($17.2 \pm 0.1\%$ headspace CO_2 after 60 h), followed by cellobiose ($12.7 \pm 0.7\%$). This difference was significant ($p = 0.012$), showing that the glucose-consuming community of fermenters and facultative anaerobes now possessed greater potential activity than the cellobiose-consuming subset. Lactate provided the third-greatest stimulation of CO_2 production ($5.1 \pm 0.1\%$), showing that anaerobic respirers of simpler substrates were also substrate-limited, while cellulose and acetate showed no stimulation of CO_2 production compared to unsupplemented controls ($\leqslant 2.6\%$; $p > 0.8$). Cellulose was therefore shown still to be sufficiently abundant after 4 weeks to supply the potential activity of cellulolytic bacteria, while acetate-consuming microbes such as methanogens were shown to have sufficiently low potential activity that their needs were also supplied adequately by indigenous acetate.

The limitation of microbial activity by the availability of cellobiose, glucose, and lactate, evident by the observation that each stimulated CO_2 production to a significantly greater extent than did cellulose ($p < 0.001$, < 0.001, and $= 0.019$ for cellobiose, glucose, and lactate, respectively) shows that cellulose hydrolysis had, after only 4 weeks, become the rate-limiting step for heterotrophic respiration, though not necessarily for sulfate reduction and/or column performance, in the system. Low CH_4 production showed that lactate and acetate were not converted into methane to great extents (compare Figs. 5a and c). The supposition that acetate and lactate simply provided smaller amounts of carbon is invalid, for the constant CO_2 production rates for all samples over the incubation period show that substrate limitation did not occur, as intentionally prevented by the design of the batch experiments.

Sulfate-reducing activity had become well established within 3 weeks (Fig. 2b) and increased greatly overall in the Day 27 batch study compared to the initial batch study ($p < 0.001$; Fig. 8b). Sulfide production was stimulated to statistically equivalent extents by cellobiose, glucose, and lactate ($p \geqslant 0.45$ for all comparisons), yielding $1.5 \pm 0.2\%$ H_2S in column headspaces after 60 h, while cellulose and acetate showed no stimulatory effect ($p > 0.999$) (Fig. 5b). Lactate is a preferred substrate for several groups of sulfate-reducing bacteria and has frequently been used to enhance their activity in passive treatment systems (Dvorak et al., 1992; Tsukamoto and Miller, 1999); these results confirmed that availability of lactate or one of its degradation products (e.g. H_2) already limited this particular sulfate-reducing community. Cellobiose and glucose, however, cannot be consumed directly by known sulfate reducers. Their abilities to stimulate sulfide production to as great an extent as lactate showed, therefore, that they were rapidly converted into forms readily available to SRB by other microbial populations. The rate-limiting step for

sulfide production was therefore upstream of each of them, at the hydrolysis of cellulose to cellobiose. While cellulose degradation appears to limit energy flow in soils and many anaerobic systems (Lynd et al., 2002), it has not previously been shown to limit SRB activity in particular in these complex systems, nor to be a specific factor affecting sulfate reduction in PRBs where multiple potential limiting parameters exist. The inability of acetate to enhance sulfide production showed that it was not a limiting substrate for sulfate reduction in these systems.

Methanogenesis was detectable in the Day 27 batch study (Fig. 5c), in contrast to the initial batch study (Figs. 4c and 8c), yielding up to 0.14% headspace CH_4. However, substrate supplements had no significant ($p = 0.598$) effect, indicating that methanogenesis rates were limited by a factor, such as a nutrient, energy source, or population size, other than these specific substrates.

3.1.4.4. Third study. Columns 1 and 7, sacrificed after 41 days of operation, were recovering from the sulfate pulse (SRR $= 0.6 \pm 0.3$ mol $SO_4^{2-}/m^3/d$; Fig. 2b) and had been removing Mn and Zn from influent for approximately 2 weeks (Fig. 3). CO_2 and H_2S production (Figs. 6a and b) were statistically equivalent ($p = 0.618$ for CO_2, $p = 0.593$ for H_2S) to the levels observed in the second batch study (Figs. 5a and b, 8a and b), however, showing that the addition of metals had not measurably affected potential activities of interest.

CO_2 production was again stimulated most by cellobiose and glucose, although at this date the effects were indistinguishable ($p = 0.120$), indicating comparable potential activities of the two communities. The effects of these two supplements were significantly greater than each of the other supplements ($p < 0.007$ for all comparisons), continuing the previous indication (Fig. 5a) that heterotrophic metabolism was limited by cellulose hydrolysis. Lactate generated slight stimulation compared to unsupplemented controls ($p = 0.026$; $4.4 \pm 0.5\%$ headspace CO_2 after 60 h), showing the comparatively small contribution of the lactate-consuming community to overall carbon flow, while acetate-supplemented, cellulose-supplemented, and unsupplemented samples were again indistinguishable ($p > 0.999$), generating $\leqslant 3\%$ CO_2, consistent with results of the second study (Fig. 5a).

Sulfate-reducing activity (Fig. 6b) was, like CO_2 production, also indistinguishable from that observed in the second study ($p = 0.678$) (Figs. 5b and 8b) and was again stimulated most, and to equivalent extents, by cellobiose and glucose ($p > 0.999$). The degraders of these substrates were therefore sufficiently active not to limit sulfate reduction, resulting in the continued position of cellulose hydrolysis as the rate-limiting step not only for total CO_2 generation, as expected in natural anaerobic systems, but also for the much smaller component of total respiration found in sulfate reduction. Lactate stimulation was slightly less but not significantly so ($p > 0.08$), consistent with the appearance of lactate and/or its degradation products as the immediate substrates for SRB in this system. Acetate and cellulose again showed no enhancement of sulfide

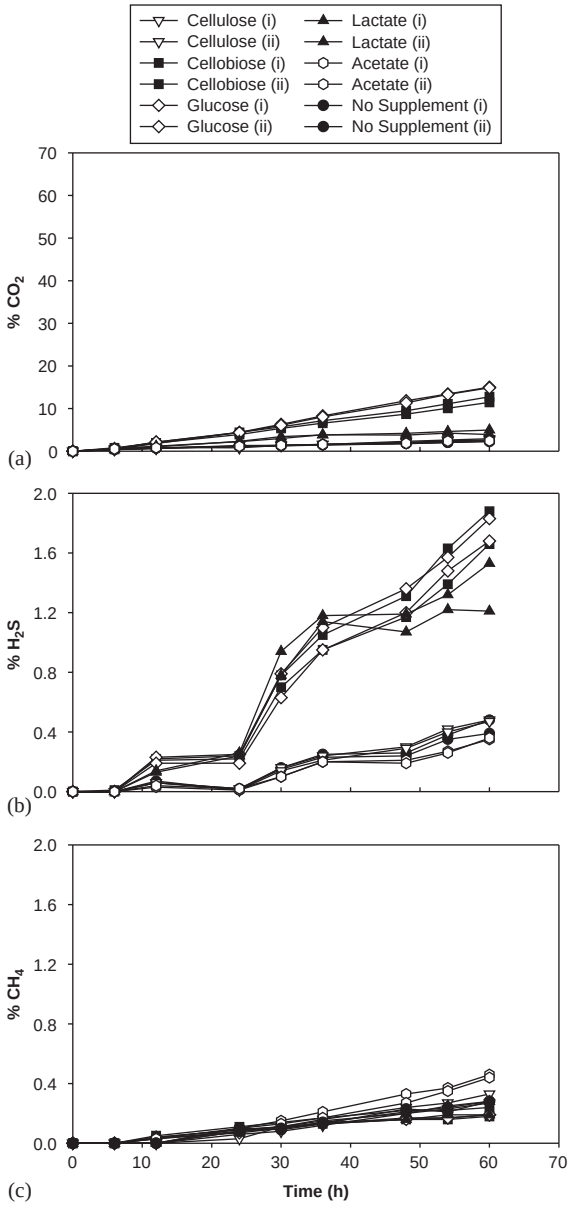

Fig. 6. Headspace molar percentages of (a) CO_2, (b) H_2S, and (c) CH_4 produced over time in Day 41 batch studies. Replicate batches consisted of sacrificed column materials supplemented with 10 mM cellulose, cellobiose, glucose, lactate, or acetate; controls received no supplements. Data points show individual measurements.

production compared to unsupplemented controls ($p > 0.95$).

Methanogenesis increased slightly in all samples (Fig. 6c) compared to the second study ($p = 0.039$; Figs. 5c and 8c), showing the rise of this population's activity. Methanogenesis also showed for the first time a small, significant stimulation by acetate over the last 12 h of incubation ($p = 0.044$), which led to the accumulation of $0.45 \pm 0.01\%$ headspace CH_4. The advent of detectable methanogenesis was therefore accompanied by near-simultaneous evidence of limitation by acetate availability. Clearly, however, the sulfate-reducing and methanogenic populations were not competing directly for either lactate or acetate. Competition between these two groups for H_2, while still likely (Qatibi et al., 1990; Raskin et al., 1996; O'Flaherty et al., 1998), appeared not to have limited sulfate-reducing activity since sulfate reduction responded vigorously to lactate supplementation, which in all likelihood generated H_2 as well (Schwartz and Friedrich, 2003), while methanogenesis did not (Figs. 8a and b).

3.1.4.5. Fourth study. Columns 3 and 4, sacrificed after 99 days of operation, were showing early signs of decline, as evidenced by their removal of significantly less sulfate ($SRR = 0.8 \pm 0.1\ mol\ SO_4^{2-}/m^3/d$; Fig. 2b) than they had as recently as Day 83 ($SRR = 1.4 \pm 0.01\ mol\ SO_4^{2-}/m^3/d$; $p < 0.001$; Fig. 2b), although Zn removal continued successfully (Fig. 3). Correspondingly, both CO_2 production (Fig. 7a) and H_2S production (Fig. 7b) in batch studies in the absence of supplements decreased relative to their counterparts in the third study ($p < 0.001$ and $p = 0.010$ for CO_2 and H_2S, respectively; Figs. 6a,b, 8a and b), indicating that the microbial capacities for both total organic carbon oxidation and sulfate reduction had declined significantly.

CO_2 production was nearly undetectable in unsupplemented controls, indicating that easily respirable organic carbon was virtually exhausted in the columns. CO_2 production in acetate- and cellulose-supplemented samples was also nearly undetectable, indistinguishable from those with no supplementation ($p > 0.999$), showing that acetate and cellulose consumption were limited by a factor other than substrate availability. Lactate provided only slight stimulation ($3.2 \pm 0.1\%$ CO_2; $p = 0.072$) compared to unsupplemented controls, in contrast to the previous sampling periods, showing that the potential activity of the lactate-consuming population (including SRB) had declined significantly. Finally, stimulation of CO_2 production was still possible (and indistinguishable; $p = 0.684$) by cellobiose and glucose supplements, but yielded only $8.8 \pm 0.6\%$ headspace CO_2 after 60 h, an amount significantly less ($p < 0.008$) than that achieved in the second and third batch studies and

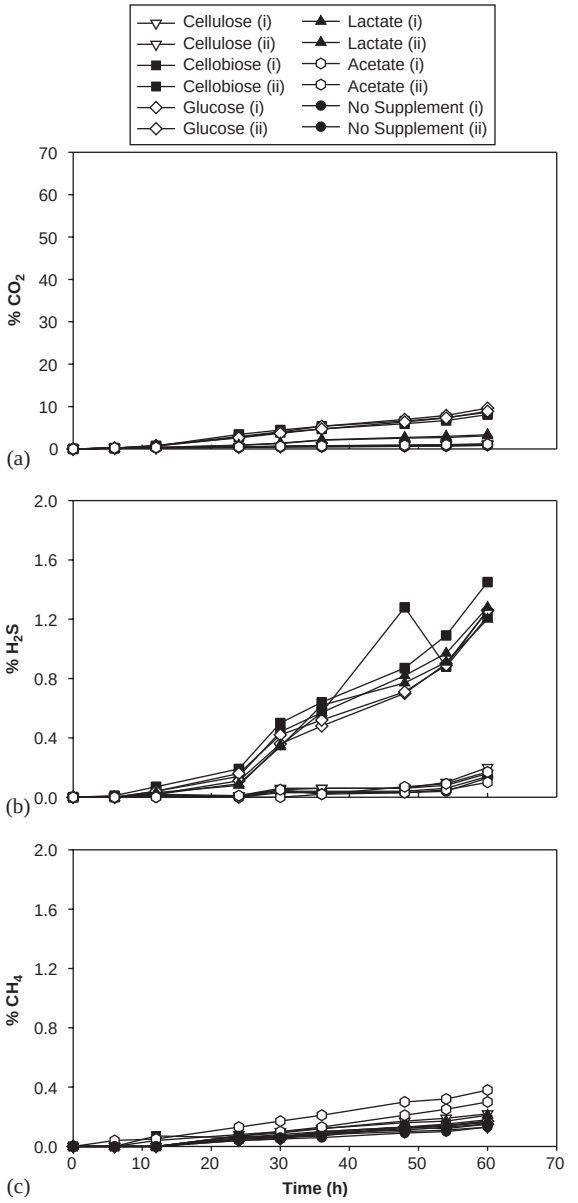

Fig. 7. Headspace molar percentages of (a) CO_2, (b) H_2S, and (c) CH_4 produced over time in Day 99 batch studies. Replicate batches consisted of sacrificed column materials supplemented with 10 mM cellulose, cellobiose, glucose, lactate, or acetate; controls received no supplements. Data points show individual measurements.

showing the decline in the glucose- and cellobiose-degrading populations.

Sulfate-reducing populations also showed decline in the fourth study. H_2S production was enhanced, to statistically equivalent extents, by cellobiose, glucose, and lactate supplements ($p > 0.460$ for all comparisons; Fig. 7b), showing that upstream populations (e.g.

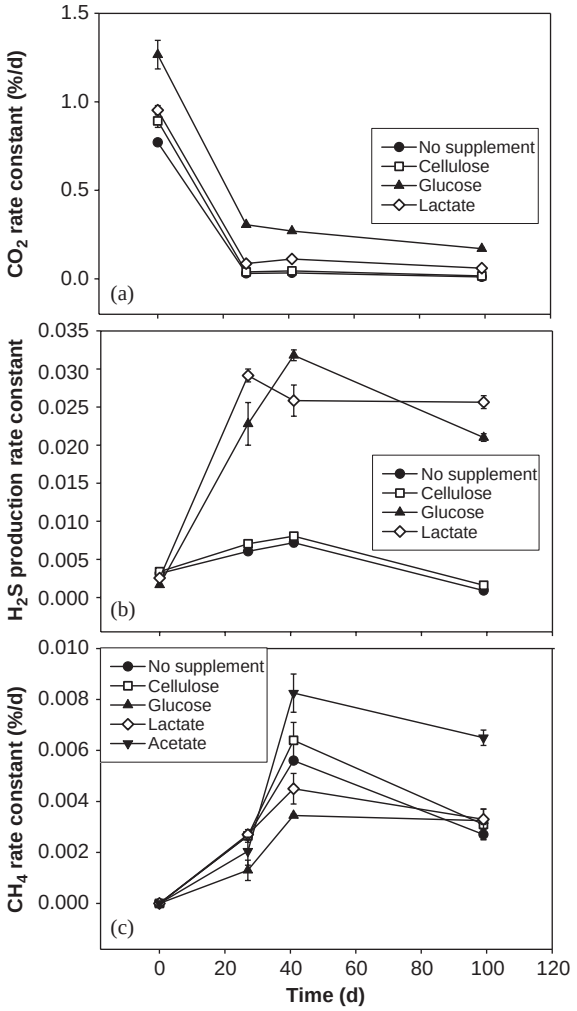

Fig. 8. Rate constants (%/d) for (a) CO_2, (b) H_2S, and (c) CH_4 production by batch studies over column lifetimes. Data points show means of duplicate measurements; error bars show the range of data.

fermenters) remained sufficiently vigorous that they still had the potential to provide non-limiting levels of substrates to the SRB. Nevertheless, H_2S headspace concentrations reached levels of only $1.3 \pm 0.1\%$, significantly lower than levels in the third study ($p = 0.031$) (Figs. 6b and 8b). Although substrate availability was still a major factor controlling sulfate reduction rates, and cellulose hydrolysis was still clearly visible as the primary step restricting substrate availability to the SRB, clearly one or more additional factors had begun to limit the potential activity of the sulfate-reducing population.

Methanogenesis in the fourth study (Fig. 7c) was indistinguishable from that in the third study ($p = 0.334$; Figs. 6c and 8c), an interesting finding considering the decline of the other populations. Only acetate showed a significant stimulatory effect, generating $0.34 \pm 0.4\%$ headspace CH_4 after 60 h. Again, a lack of substrate competition between SRB and methanogens was evident, an important finding in light of the known possibility of such competition in other anoxic environments (Capone and Kiene, 1988; Lovley and Goodwin, 1988) and one that allows future PRB design to focus on the roles of microbial communities upstream of the SRB.

3.1.5. Microbial community dynamics

Batch study results elucidated two important and related factors influencing PRB performance: first, the substrate availability for each member of the microbial community, and second, the potential activity of each community member independent of substrate availability. To examine the second factor more closely, the linear portion of each batch study gas production curve was used to compute a characteristic gas production rate. These rates were then compared over the lifetimes of the columns (Figs. 8a–c). The overall heterotrophic (CO_2-producing) potential of the communities diminished strikingly from Days 0 to 27 (Fig. 8a). This potential can be considered a proxy for the population size if it is assumed that minimally active microbes regained activity during the batch studies: the heterotrophic population thus appeared to have diminished substantially within the first weeks of column operation. In contrast, however, the sulfate-reducing (H_2S-producing) potential activity (population) rose from Days 0 to 27 and then remained present and highly responsive to glucose and lactate through Day 99 (Fig. 8b), showing that declines in the activity of upstream populations, perhaps due to lack of substrate availability rather than declines in the SRB population, were likely to have caused the decline of sulfate-reducing activity. Methanogens also maintained half or more of their maximal potential activity through Day 99 (Fig. 8c), especially in response to acetate, showing the continuing vitality of the methanogen population despite declining substrate availability.

4. Conclusions

The accumulation of reports from pilot PRBs detailing performance decline has proven that further understanding of system processes is essential to the design of effective barriers. In our investigation of one potential category of problems, the "depletion of the reactive component" (Blowes et al., 2000), we elucidated the interactions of four important microbial communities (cellulolytics, fermenters + respirers, sulfate reducers, and methanogens) with each other and with their environment throughout the establishment, performance,

and decline of laboratory-scale PRBs. Several key results thus emerged that have direct bearing on the design of PRBs as well as on other treatment systems employing anaerobic biozones.

First, the cellulolytic community was limited at no time by cellulose availability, indicating that its activity is independent of the quantity of cellulosic substrate provided. Instead, if enhancement of cellulolysis is desired, other measures must be taken, potentially including the provision of pre-treated biomass with reduced cellulose crystallinity, degree of polymerization, particle size, and/or extent of lignification, as well as increased accessible surface area (Lynd et al., 2002). The seeding of PRB materials with cellulolytic bacteria and fungi is another option worthy of exploration, especially in light of the development of improved cellulose degraders, as is the provision of molecular inducers of cellulase production, such as sophorose (Lynd et al., 2002). Cellulolysis is also highly sensitive to pH values below 6.0, indicating that careful effort must be expended to ensure that influent pH is raised to that level before it reaches the desired zone of cellulose decomposition (Lynd et al., 2002).

Second, the fermentative and respiratory community maintained vigor throughout the column operation, even during performance decline, and was able to stimulate sulfate reduction indirectly with great effectiveness. While provision of glucose or another readily fermentable carbon source to a failing PRB would be an expensive course of action, it nevertheless emerges as an attractive option to maintain performance temporarily while other troubles are addressed.

Third, the sulfate-reducing community showed an unexpectedly great limitation by substrate availability through all but the early establishment phase. The severity of this limitation, and the reversal provided by supplementation, showed convincingly that nutrient availability, sulfate availability, metal toxicity, and pH considerations played only minor, if any, roles in limiting the SRB component of column performance. Activities of fermenters and respirers, downstream of cellulose degradation, were also shown not to limit SRB activity, with the result that cellulolysis emerged as an even more important factor in PRB performance. The importance of exploring means by which to enhance cellulolysis therefore increased concomitantly. While cellulose is known to be a relatively recalcitrant substrate, its rate of decomposition cannot be assumed to limit downstream activities in anaerobic systems, particularly in complex communities (Bruechert and Arnosti, 2003). The unambiguous nature of this result is therefore quite useful in clarifying priorities, from the perspective of the SRB, in optimizing system performance.

Fourth, lactate emerged as a strongly preferred substrate, in comparison to acetate, for SRB; this suggested low levels of competition between SRB and acetate-consuming populations and indicated that acetate would not be a useful substrate to revive a declining PRB.

Fifth, competition by methanogenesis was, at most, a minor factor affecting sulfate reduction rates. Methanogenesis, in contrast to all other activities, was not limited by cellulolysis, though it was limited at later timepoints by acetate availability. The lack of methanogenic competition with SRB for lactate or its degradation products (such as H_2) shows that measures to limit methanogenesis in PRBs are probably unnecessary, and that competition from other lactate- and H_2-consuming anaerobic respirers (e.g. iron and manganese reducers) is likely to be much more important.

Two primary approaches have been explored in the application of anaerobic biozones to sulfate reduction and metal removal. In one, the goal is to accelerate the rate of decomposition by providing small distinct reaction zones to allow high throughput, with the drawback that organic substrates must be added frequently (Pulles and Rose, 2002). In the other, the goal is to discover combinations of organic substrates that, in comparatively large quantity, promote longer-term, lower-rate operation (Waybrant et al., 1998; Blowes et al., 2000). Both approaches, as well as others using anaerobic biozones for sulfate and metal removal, depend fundamentally on microbial community dynamics. In all cases, appropriate design necessitates that the performance be predictable and to some extent controllable. Predictability and control, in turn, require that factors dictating SRB activity be well understood. This study illustrates the central importance of microbial population activities in limiting SRB activity, as well as the evolution of these activities over time, allowing future PRBs and other passive systems to be designed and operated with greater insight into the underlying microbial processes.

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